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Hepatitis C Virus Structural Proteins Induce Liver Cell Injury in Transgenic Mice

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To develop an animal model of hepatitis C virus (HCV) infection, transgenic mice carrying part of the HCV cDNA (C980) encoding HCV-core and envelope proteins under control of the mouse class I major histocompatibility complex gene (H-2K) regulatory region were produced. HCV-C980 RNA and HCV-core protein were present in livers from line H36 as determined by RNase protection assay and immunostaining, respectively. More than 40 animals from line H36 were examined histologically. Most of these H36 mice after 10 months of age developed spontaneous focal infiltration of lymphocytes, hepatocyte necrosis, degeneration, and altered foci with mitotic hepatocytes. These pathological lesions were absent in livers from the age-matched control littermates. Liver cells from these H36 mice were sensitive to damage induced by intravenous administration of an anti-Fas antibody. It is suggested that HCV-C980 proteins by themselves may be one causative agent of liver cell injury in subjects with HCV infection. ***J. Med. Virol. 59:281–289, 1999.***

KEY WORDS: apoptosis; Fas; hepatitis C virus; transgenic mice

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of hepatitis [Choo et al., 1989; Kuo et al., 1989; Houghton et al., 1991]. At least 50% of infected persons develop chronic hepatitis, and in 10–20% cirrhosis occurs. HCV is associated frequently with the development of hepatocellular carcinoma (HCC) [Saito et al., 1990; Gerber, 1993]. HCV has a single-stranded RNA genome of positive polarity, about 9,500 nucleotides in length, with a single large open reading frame that encodes a precursor polyprotein of 3,010, 3,011, or 3,033 amino acid residues (variations depending on the genotype) [Kato et al., 1990; Choo et al., 1991; Takamizawa et al., 1991]. The polyprotein is cleaved co- and posttranslationally into putative structural and nonstructural proteins [Hijikata et al., 1991; Grakoui et al., 1993; Selby et al., 1993]: NH_2-core (C)-envelope 1 (E1)-p7-envelope 2 (E2)-nonstructural protein 2 (NS2)-NS3-NS4A-NS4B-NS5A-NS5B-COOH. Using an in vitro processing system, three putative viral structural proteins, p22 (C), gp35 (E1), and gp70 (E2), are processed possibly by a signal peptidase of host cells [Hijikata et al., 1991], whereas HCV-encoded metalloproteinase and serine proteinase, located in NS2 to NS3 region and *N*-terminal one-third of NS3 protein, respectively, mediate cleavages of other sites in the nonstructural region [Hijikata et al., 1993].

Mechanisms related to the pathology that allow for persistence of infection and result in high rate of chronic liver disease, including HCC, are not well understood. Chronicity of infection as well as histopathologic findings suggest that liver disease may be mediated by immune mechanisms. Prospective studies on immune responses to HCV have shown that antibodies to the viral nucleoprotein usually appear within 10

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weeks from clinical onset [Hosein et al., 1991]. Moreover, a transient IgM anti-HCV-core response has been observed in patients with acute posttransfusion hepatitis C and with acute exacerbation of their chronic liver disease [Chen et al., 1992] and in the HCV cDNA transgenic mice [Wakita et al., 1998], suggesting that HCV-core protein is an important target for immune responses. This concept is supported further by recent findings that HCV-core protein induces vigorous T-lymphocyte proliferative responses in patients with chronic HCV infection [Ferrari et al., 1994; Diepolder et al., 1996; Leroux-Roels et al., 1996; Rehermann et al., 1996]. Furthermore, HCV-specific cytotoxic T lymphocytes were also identified both in hepatic parenchyma [Koziel et al., 1992] and in peripheral blood [Kita et al., 1993] of patients with HCV infection. These T cells recognized epitopes in HCV structural proteins.

An animal model of HCV infection was produced by generating transgenic mice carrying part of the HCV cDNA (C980) encoding HCV-core and envelope proteins under control of the promoter of the class I major histocompatibility complex (H2-K) gene. Stable expression of HCV-C980 RNA and HCV-core protein was evident in livers from one of the transgenic lines (H36). With no transfer of immune cells into the transgenic mice, coagulation necrosis of hepatocytes and focal lymphocyte infiltration occurred in the transgenic mice after 10 months of age. The liver cells were sensitive to damage induced by intravenous administration of an anti-Fas antibody. The pathogenesis of liver cell injury caused by HCV-C980 proteins is discussed.

MATERIALS AND METHODS

Construction of H2-C980 Plasmids

The H2-C980 transgene was constructed as follows: A 3.0-kb *Stu*I fragment containing the coding region of HCV(1b) structural proteins (C, E1, E2) was isolated from pC980 plasmid [Hijikata et al., 1991]. The fragment was ligated to *Hin*dIII linker and inserted into the *Hin*dIII site of plasmid pH2/4 that had the H2-K gene promoter, the rabbit β-globin genomic sequence, and the SV40-derived polyadenylation signal [Nishi et al., 1988], resulting in construction of the plasmid pH2-C980 (Fig. 1A). pH2-C980 was digested with *Pvu*II and *Sal*I, and a resulting 4.6-kb fragment was isolated and used for microinjection.

DNA Injection and Screening of Transgenic Mice

C57BL/6CrSlc and ICR mice were purchased from Japan SLC Co. (Hamamatsu, Japan) and Japan CLEA Co. (Tokyo, Japan), respectively. Transgenic mice were produced by the method described by Hogan et al. [1986]. Briefly, C57BL/6 mice were used to obtain fertilized eggs, and the H2-C980 fragments were microinjected into a male pronucleus of fertilized eggs. The injected eggs were returned to the oviducts of pseudopregnant mothers of ICR strain. When the mice were 4 weeks of age, total DNA was extracted from a tail of each mouse. The exogenous DNA in tail DNA from

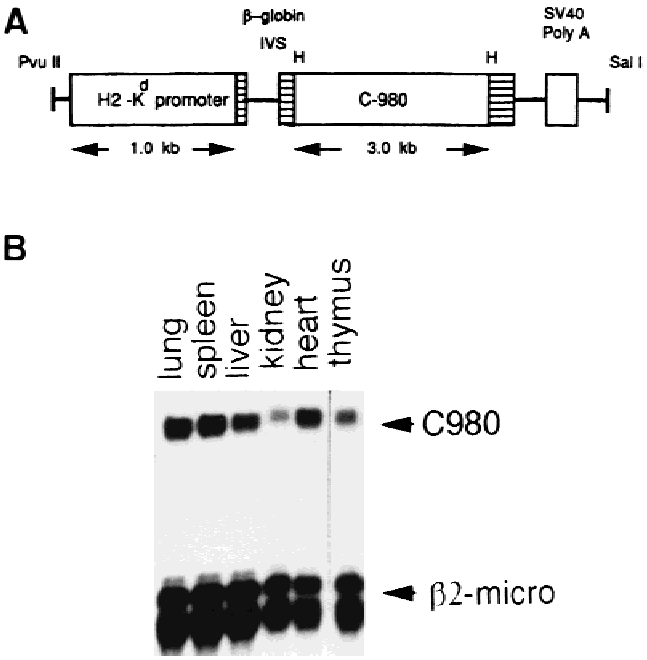

Fig. 1. H2-C980 transgenic mice. **(A)** An injection fragment of the H2-C980 gene. **(B)** Expression of HCV-C980 RNA in various tissues of H36 mice. The level of C980 RNA was measured by RNase protection assay. The expected 172-bp band of C980 RNA was detected. The level of β2-microglobulin (β2-micro) RNA was used as an amount control of RNA.

transgenic mice was detected by Southern blots, as described previously [Yamamoto et al., 1995]. Briefly, 50 μg of tail DNA were transferred to a nylon membrane (Hybond N, Amersham International, Buckinghamshire, UK) and fixed by cross-linked with ultraviolet irradiation using Spectrolinker (Schleicher and Schuell, Dassel, Germany) and by baking at 120°C for 30 min. The filter was prehybridized for 1 hr and hybridized overnight at 42°C in 50% formamide hybridization buffer with 0.02% sodium dodecyl sulfate (SDS), 0.1% salcocine, 1% blocking reagent, and 10 ng/ml of digoxigenin-labeled probe. Following hybridization, the filter was washed twice for 5 min with 2× SSC and 0.1% SDS at room temperature and twice for 15 min with 0.1% SSC and 0.1% SDS at 68°C. The digoxigenin-labeled probe was detected with sheep anti-digoxigenin antibody conjugated with alkaline phosphatase. The antibody detection reaction was carried out using an enhanced chemiluminescent detection system (Boehringer Mannheim, Mannheim, Germany). For the probe, a 575-bp (*Stu*I-*Xba*I) fragment containing the core region of HCV cDNA into pGEM5Z(−) plasmid was labeled by digoxigenin (Boehringer Mannheim) using the polymerase chain reaction with T7 and SP6 primers [Fukuda et al., 1997].

RNase Protection Assays

Total RNAs were extracted from adult mouse tissues by the acid-guanidine isothiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. HCV RNA and β2-microglobulin RNA were detected by

RNase protection assay [Ochi et al., 1994]. Briefly, total RNA (10 μg) was hybridized overnight with ^{32}P-labeled RNA probe at 55°C in 80% formamide hybridization buffer, followed by digestion with RNaseA and RNaseT1 (Sigma Chemical Co., St. Louis, MO) at 37°C for 1 hr. Protected fragments were separated by electrophoresis in a 6% polyacrylamide-urea denaturing gel. The dried gel was exposed to Kodak X-Omat AR X-ray films for 2 days at −70°C. The following DNAs were used as a template to generate anti-sense RNA probes by SP6 RNA polymerase (Promega, Madison, WI). HCV-E2: a 172-bp *Bam*HI fragment of the E2 region of HCV cDNA was subcloned into the *Bam*HI site of pGEM4Z plasmid; β2-microglobulin: a 950-bp *Hin*dIII-*Eco*RI fragment including the 238-bp mouse β2-microglobulin gene [Parnes and Seidman, 1982] was subcloned into the *Hin*dIII-*Eco*RI site of pGEM4Z plasmid.

Quantitative Analysis of HCV-Core Protein

The quantity of HCV-core protein in various tissues from those transgenic mice was measured by a sensitive fluorescence enzyme immunoassay (FEIA) [Kashiwakuma et al., 1996] with a slight modification. Briefly, the 5F11 monoclonal anti-HCV-core antibody was used as the first antibody to solid phase, and the 5E3 antibody conjugated with horseradish peroxidase was for the second antibody. This FEIA can detect as little as 4 pg/ml of recombinant HCV-core protein.

Immunohistochemistry

The highly active antibody to HCV-core protein was raised in a rabbit. The entire region of HCV-core protein derived from HCV-J belonging to HCV-1b was expressed in *Escherichia coli* [Muraiso et al., 1990] and purified to homogeneity by column chromatography [Muraiso et al., 1991]. A 2,000 dilution of the obtained serum could detect specifically HCV-core protein using Western blot analysis.

Liver tissues were fixed in 5% formaldehyde solution for 7 days. Sections (5 μm thick) of paraffin-embedded tissues were stained with hematoxylin and eosin. Intrahepatic distribution of HCV-core protein and α-fetoprotein (AFP) was assessed by the indirect immunoperoxidase method [Chisari et al., 1989]. Briefly, sections were reacted with the rabbit anti-HCV-core antibody, the anti-AFP antibody (ICN Biomedicals, Cost Mesa, CA), or normal rabbit serum as a primary antibody for 1 hr. For detection of the primary antibody, a biotinylated anti-rabbit IgG antibody (ICN) followed by avidin-peroxidase (ICN) was used.

Serum Transaminase Assay

A monoclonal anti-Fas antibody (Jo2; PharMingen, San Diego, CA) was injected intravenously into transgenic mice. Blood samples were collected 24 hr after injection. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in serum were measured using the Lippi-Guidi's method (Iatrozyme TA-LQ; Dia-Iatron, Tokyo, Japan) [Lippi and Guidi, 1970]. Mean values (and SD) of both ALT and AST in serum of H36 mice 24 hr after injection were compared with those of control littermates using unpaired Student's *t*-test.

RESULTS

Expression of the HCV Gene in Livers From Transgenic Mice

Lines of H2-C980 transgenic mice were developed from four founder (H28, H33, H35, and H36) mice. Expression of the HCV-core RNA was examined in total RNA from various tissues of these mice using Northern blots. Expression was not detected in livers from four lines of H2-C980 mice. Expression of the H2-C980 gene was examined further in livers from four lines of H2-C980 mice using the more sensitive RNase protection assay with anti-sense HCV-envelope RNA as a probe. HCV RNA was detected in livers only from line H36 and was also detected in lung, spleen, kidney, heart, and thymus from H36 mice (Fig. 1B). The quantity of HCV-core protein in liver, thymus, and spleen from H36 mice was measured by FEIA [Kashiwakuma et al., 1996]. Liver, thymus, and spleen from H36 mice expressed 0.27, 0.78, and 1.1 ng/mg of protein, respectively.

Translation of HCV RNA in the liver was also examined immunohistochemically (Fig. 2). Paraffin sections of livers from H36 mice were stained with a rabbit anti-HCV-core antibody. Hepatocytes located around the central veins gave a strong positive staining reaction, the control mouse liver did not stain with the anti-HCV-core antibody, and the H36 liver showed no staining with normal rabbit serum (data not shown). Therefore, progeny of line H36 from H2-C980 mice were used for further characterization.

Pathological Changes in Livers From Naive H36 Mice

Histopathological analysis of hematoxylin and eosin stained tissue sections was undertaken. H36 mice were killed periodically between the age of 4 and 20 months; as matched controls, nontransgenic littermates were killed at the same time points. More than 40 animals of H36 line were examined. Most of the livers from H36 mice before 6 months of age appeared normal (data not shown). Lesions such as infiltration of lymphocytes, hepatocyte necrosis, degeneration, and hepatocellular altered foci were frequent in livers from H36 mice after 10 months of age. Thus, livers were examined from 10 mice of the H36 line and 9 mice of control littermates between 10 and 15 months of age (Fig. 3). Infiltration of lymphocytes surrounding several necrotic hepatocytes was frequent around the central veins and/or in lobules of the liver from 8 of the 10 mice of H36 (Fig. 3A). Focal coagulation necrosis of hepatocytes with infiltration of lymphocytes and neutrophils was also detected in livers from 5 of the 10 H36 mice (Fig. 3B); hepatocytes located outside these foci were normal. An altered focus with mitotic hepatocytes, acidophilic inclusion bodies, and fatty change were present in livers from 5 of the 10

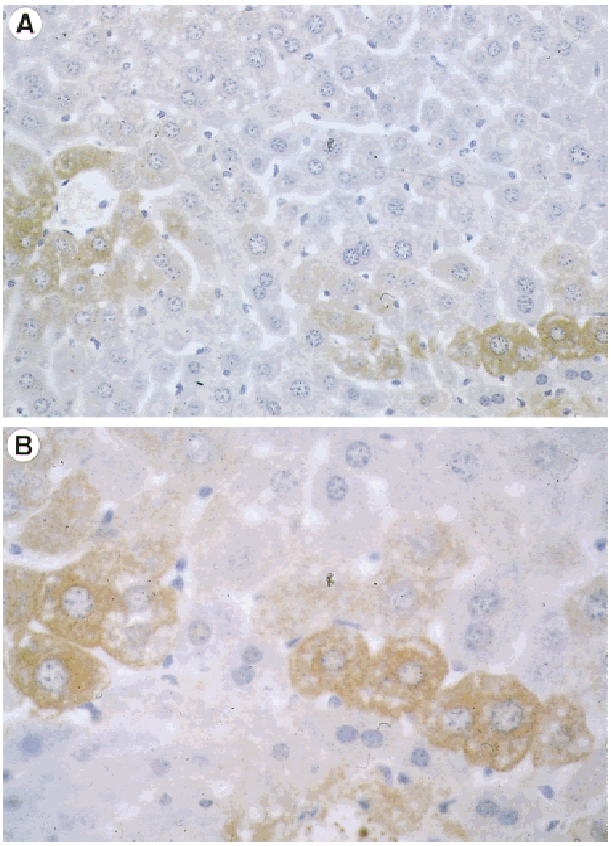

Fig. 2. Expression of the hepatitis C virus (HCV)-core protein in hepatocytes of H2-C980 transgenic mice. Liver tissue of the H36 line at 10 months of age was stained with anti-core antiserum. **(A)** Positive immunoreaction for HCV-core protein within some hepatocytes mainly in the centrolobular zones (×250). **(B)** Demonstration of HCV-core protein within the cytoplasm with a higher magnification of (A) (×320).

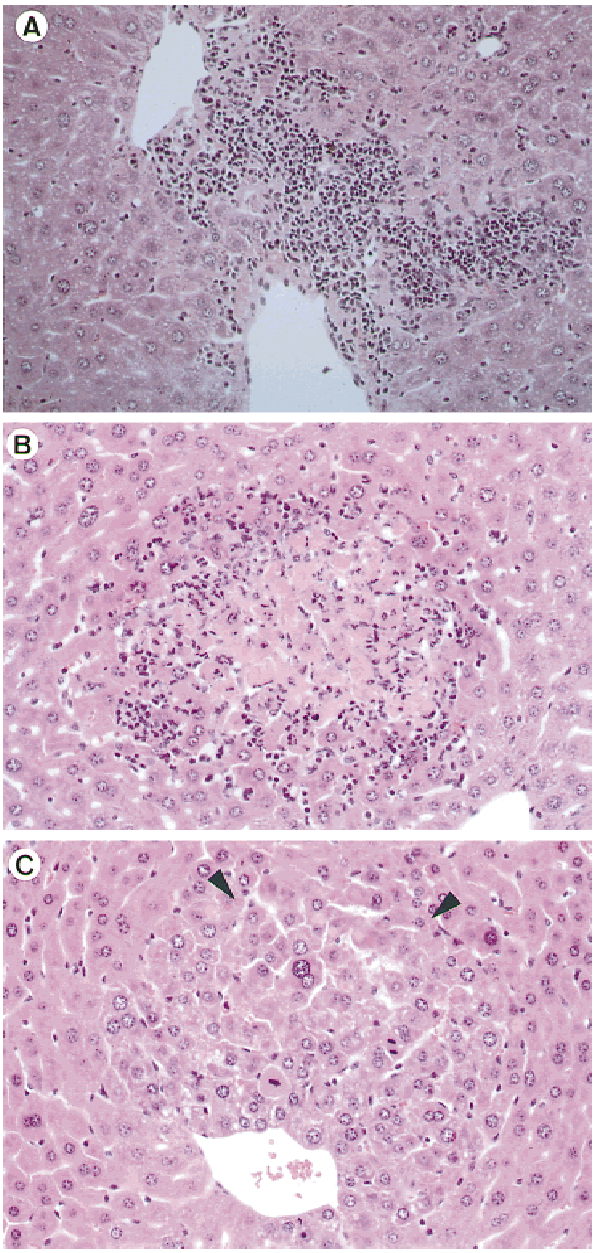

Fig. 3. Histological analysis of liver from naive H36 mice. Sections of liver from H36 mice at 10 months of age were stained with hematoxylin and eosin. **(A)** Marked infiltration of lymphocytes in hepatic centrolobular zones (×125). **(B)** Focal coagulation necrosis of hepatocytes with infiltration of lymphocytes, neutrophils, and macrophages (×165). **(C)** Discrete altered focus comprising basophilic hepatocytes with mitotic figures and acidophilic bodies (arrowed). The focus is apparently proliferated and compressing surrounding hepatic parenchyma (×165).

mice of H36 (Fig. 3C). These hepatocytes were basophilic and proliferated to compress neighboring normal liver parenchyma. These pathological lesions were absent in all livers from 9 control littermates (data not shown).

As these pathological lesions in liver sections from H36 mice (Fig. 3) suggested the presence of AFP, a marker of hepatocellular regeneration and transformation [Sell et al., 1989], liver sections were stained with a rabbit anti-AFP antibody. AFP was detected strongly in the fetal liver as a positive control (Fig. 4A). Hepatocytes located around central veins (Fig. 4B) in the livers from H36 mice were intensely positive for AFP, but AFP was absent in livers from control littermates (Fig. 4C). However, neoplastic nodule or a carcinoma did not occur in livers from H36 mice during 4 years of observation.

Fas-Mediated Liver Cell Injury in Transgenic Mice

Because an intraperitoneal injection with 10–100 μg of the anti-Fas antibody (Jo2) into normal mice induces fatal liver damage within 8 hr after injection [Ogasawara et al., 1993], susceptibility of Fas-mediated apoptosis in liver cells from H36 mice was examined after injection of the Jo2 antibody. To determine the amount of antibody that would induce a moderate level of liver cell injury in normal mice, several doses of the antibody were injected intravenously into normal mice between 8 and 10 weeks of age. Liver cell injury was determined by measuring both ALT and AST in serum from the mice 24 hr after the injection. As shown in Figure 5A,

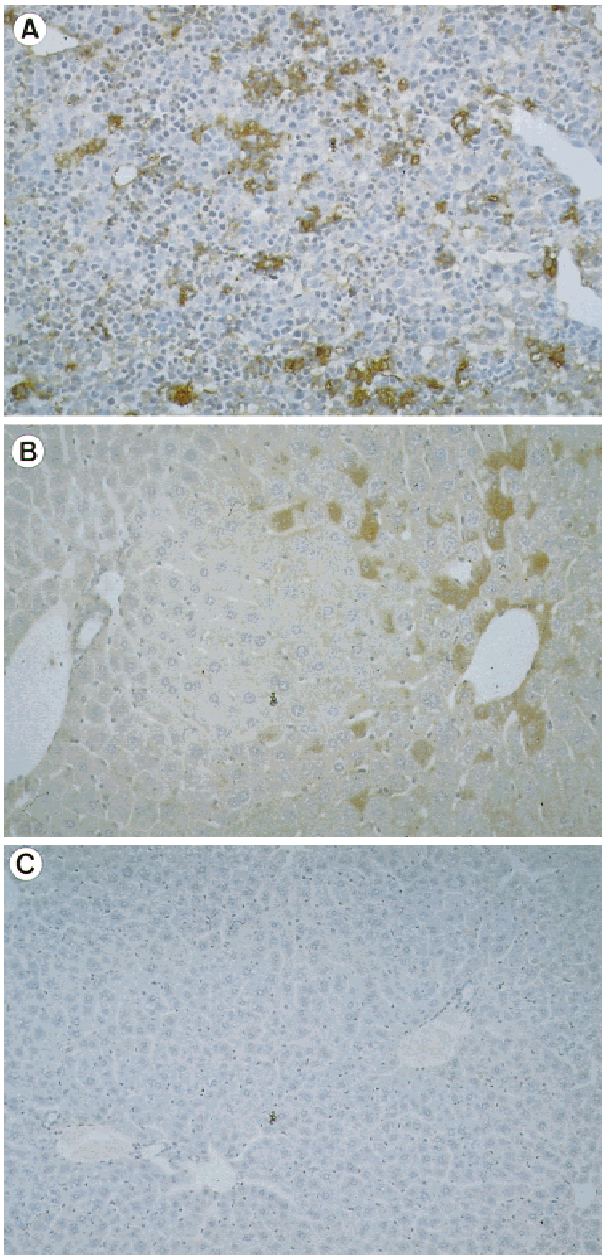

Fig. 4. Production of α-fetoprotein (AFP) in liver cells from naive H36 mice. Liver tissues of fetus (embryonal day 18), H36 line, and control littermate at 10 months of age were stained with an anti-AFP antibody. **(A)** AFP-positive liver cells scattered in fetal liver of control mouse (×165). **(B)** Some centrolobular hepatocytes from H36 mice intensely positive for AFP (×125). **(C)** No immunoreaction for AFP in control mouse liver (×85).

the activity of ALT was less than 40 U/L in serum before the injection. When mice were injected with more than 2 μg of the antibody, liver cell injury was detectable. Injection of over 4 μg of the antibody led to death of the mice within 12 hr after the injection (data not shown). Because the activity of ALT was about 300 U/L in serum from mice injected with 2 μg of the antibody, kinetics of the liver cell injury was analyzed further. The activity showed a peak at 12 hr after injection and gradually decreased at 24 hr after injection (Fig. 5B). The activity of AST was similar to that of ALT in those experiments (data not shown). Thus, H36 mice were injected with 2 μg of the antibody and activities of ALT and AST in serum were measured 24 hr later (Fig. 6). The activities were less than 40 U/L in serum from H36 mice before the injection. As the activities from H36 mice were higher than in control littermates, liver cells from H36 mice were more susceptible to damage.

Liver injury from those transgenic mice 24 hr after injection of Jo2 antibody was confirmed histologically (Fig. 7). Livers from H36 mice (ALT: 10,064 U/L) showed many areas of focal hemorrhage and necrosis around central veins (Fig. 7A). Because livers from control littermates (ALT: 413 U/L) (Fig. 7B) showed no evident abnormalities, the severity of liver injury correlated with activity of ALT and AST in the serum.

DISCUSSION

Most of the naive H36 mice had liver lesions such as infiltration of lymphocytes, hepatocyte necrosis, and altered foci with mitotic hepatocytes around the hepatic central veins, all of which occurred in mice after 10 months of age. Although positional effects (insertional mutagenesis) of the exogenous gene in H36 mice cannot be determined based on results from one mouse line, it may be possible that HCV-C980 proteins may cause these lesions. The H2-C980 gene encodes three major viral structural proteins (C, E1, E2) of HCV. Production of structural proteins (C, E1, E2) from HCV precursor polyprotein is mediated by proteolytic cleavage by signal peptidase(s) of endoplasmic reticulum lumen in host cells [Hijikata et al., 1991]. The HCV-C980 precursor polyprotein can be processed to three major viral proteins in hepatocytes of H36 mice. Because transgenic mice expressing HCV-E1 and E2 protein in liver cells did not reveal pathological changes in liver up to 16 months of age [Koike et al., 1995], the lesions in naive H36 mice may not be caused by HCV-E1 and E2 protein alone. It may be that the HCV-core protein or a combination of HCV-core and envelope proteins cause the pathological lesions in livers from H36 mice.

Pathogenesis of liver lesions from naive H36 mice may be explained by a higher sensitivity of liver cells to Fas-mediated liver cell injury. This notion of a higher susceptibility is supported by results from in vitro gene transfection of HCV structural genes into a human hepatoblastoma cell line, HepG2 [Ruggieri et al., 1997]. Although HepG2 without transfection is insensitive to stimulation with an anti-Fas antibody, Fas-mediated apoptosis is induced in HepG2 transfected with the HCV-core gene. Overexpression of HCV-envelope proteins without HCV-core protein in HepG2 did not enhance such Fas-mediated apoptosis [Ruggieri et al., 1997], suggesting that the HCV-core protein or a combination of HCV-core and envelope proteins as effective agents for Fas-mediated liver cell injury. A specific immune reaction to hepatocytes such as infiltration of lymphocytes in the lesion may occur after the injury.

HCV structural proteins may enhance susceptibility

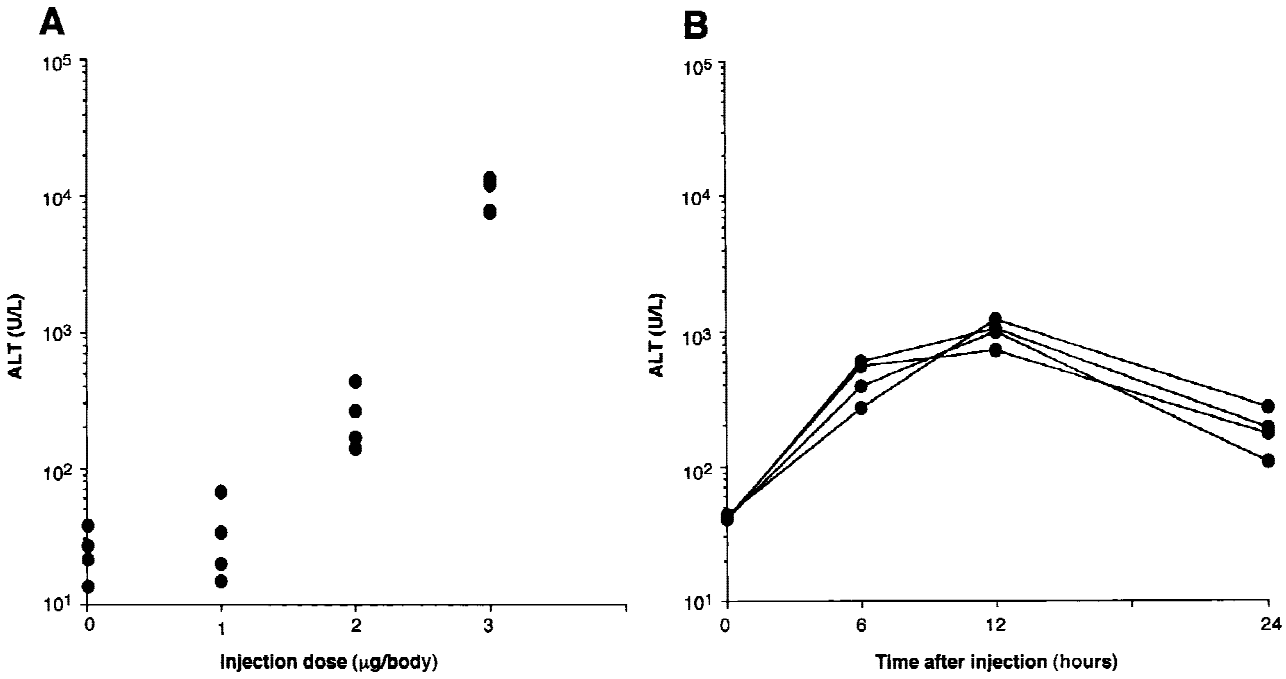

Fig. 5. Liver cell injury mediated by an anti-Fas antibody in normal mice. **(A)** Several doses of the Jo2 antibody were injected intravenously into normal mice. Activity of alanine aminotransferase (ALT) was measured in serum 24 hr after injection. **(B)** The Jo2 antibody (2 μg) was given to normal mice intravenously and the activity of ALT in serum was measured several hours after the injection. Each circle indicates an individual mouse.

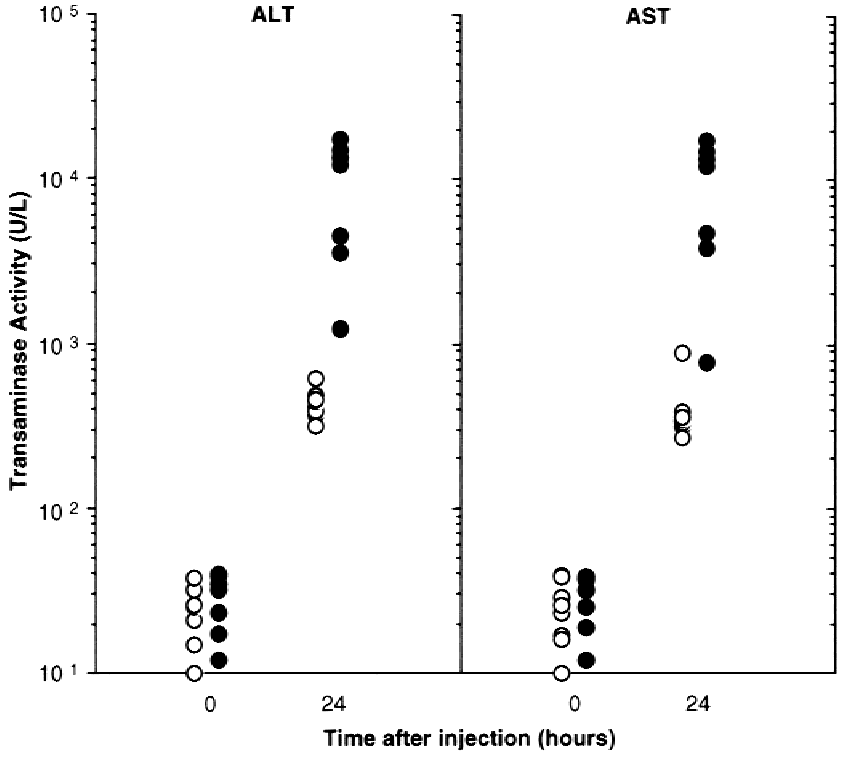

Fig. 6. Liver cell injury mediated by an anti-Fas antibody in H36 mice. The Jo2 antibody (2 μg) was given intravenously to H36 mice and the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum was measured 24 hr after the injection. Each circle indicates an individual mouse. ●, H36 mice; ○, control littermates. Activity of both ALT and AST in serum from H36 mice and control littermates before injection of the Jo2 antibody was less than 40 U/L. Statistical analysis of ALT and AST in sera between H36 and control littermates 24 hr after injection indicates $P < .005$.

to Fas-mediated apoptosis in liver cells. Because the level of Fas antigen on HepG2 transfected with the HCV-core gene was not augmented [Ruggieri et al., 1997], HCV-core protein may enhance the Fas-mediated signal transduction pathway in HepG2. The level of Fas antigen on liver cells from transgenic mice was hardly comparable with that from control littermates as determined immunohistochemically. When

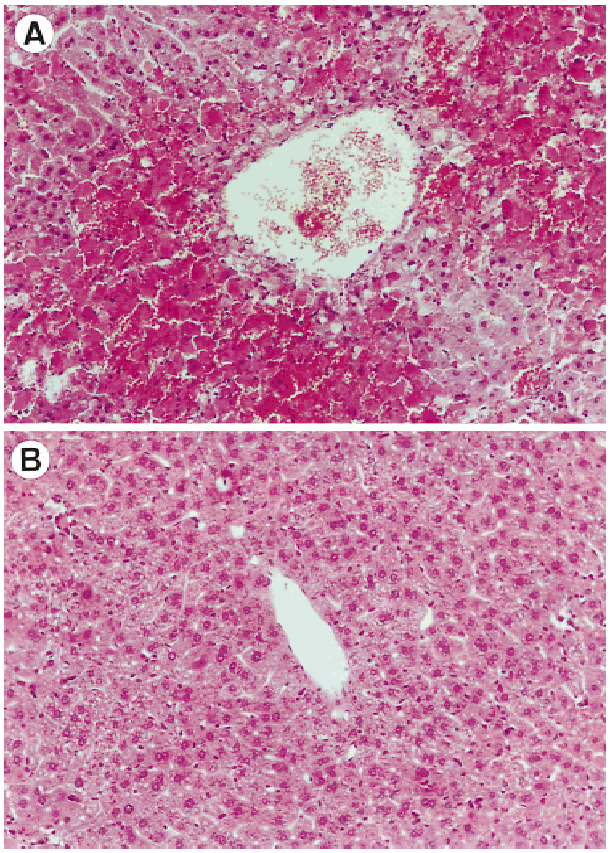

Fig. 7. Histological analysis of Fas-mediated liver cell injury in H36 mice. The Jo2 antibody (2 μg) was given intravenously to H36 mice and liver sections from these mice were examined 24 hr later. **(A)** Liver from a H36 mouse with alanine aminotransferase (ALT): 10,064 U/L, aspartate aminotransferase (AST): 16,570 U/L. Note focal hemorrhage and necrosis around the central veins (×125). **(B)** Liver from a control littermate with ALT: 493 U/L, AST: 332 U/L. Note the lack of abnormality (×125).

the amount of Fas antigen was examined in thymocytes and spleen cells from H36 mice by flow cytometry, the level was not augmented (data not shown), which suggests that HCV structural proteins augment the signal transduction pathway initiated by Fas in liver cells. Furthermore, spontaneous hepatic necroinflammatory changes that occurred in H36 mice with the small amount of HCV structural proteins may explain why most HCV carriers with persistently normal ALT showed mild inflammatory changes in liver [Naito et al., 1994], although moderate to severe hepatitis in HCV infection may be mainly immune mediated [Ferrari et al., 1994; Diepolder et al., 1996; Leroux-Roels et al., 1996; Rehermann et al., 1996].

In naive H36 mice, hepatocyte necrosis, hepatocellular altered foci, and the production of AFP in liver cells occurred in mice after 10 months of age. Altered foci and the production of AFP are considered as evidence of a preneoplastic lesion [Hirota et al., 1982; Hirota and Williams, 1989; Lee et al., 1990; Kim et al., 1991], suggesting that HCV structural proteins play a role in carcinogenesis. Indeed, expression of HCV-core protein in the liver induces hepatic steatosis [Moriya et al., 1997] and HCC in transgenic mice [Moriya et al., 1998]. However, no neoplastic or cancerous lesions occurred in liver from H36 mice by the age of 20 months. Because the truncated form of HCV-core protein can enter the nucleus [Ravaggi et al., 1994; Suzuki et al., 1995], some HCV-core protein in H36 mice may be cleaved, enter the nucleus, and affect expression of the endogenous gene to express carcinogenic activity like HBVX protein [Shirakata et al., 1989]. Why a frank neoplasm did not appear might relate to the level of liver cell injury in the H36 mice. Chronic hepatitis and cirrhosis usually precede development of HCC in HCV-infected human liver [Saito et al., 1990; Gerber, 1993], suggesting that even a small amount of HCV-core protein may advance from preneoplastic lesions in cirrhosis to HCC in human livers.

The extrahepatic manifestations accompanying human HCV infection such as Sjøgren's syndrome [Haddad et al., 1992], glomerulonephritis [Johnson et al., 1993], or cryoglobulinaemia [Agnello et al., 1992] were also examined. However, no abnormality was detected in related tissues from those transgenic mice (data not shown), suggesting that immune-reactive cells are likely to be required for the development of these abnormalities. Because a correlation between carcinogenesis and HCV infection has also been suggested in case of tissues other than the liver, for example a subset of B-cell lymphomas [Mazzaro et al., 1996; De Vita et al., 1997], those transgenic mice will be useful to examine roles of HCV structural proteins in not only chronic hepatitis but also in cases of extrahepatic lesions in human HCV infections.

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